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Paper

Toxicity assessment and DNA repair kinetics in HEK293 cells exposed to environmentally relevant concentrations of Glyphosate (Roundup® Control Max)

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Glyphosate is a systemic, non-selective, pre and post-emergence wide range herbicide. In 2015, IARC classified Glyphosate as "a probable carcinogenic agent for humans". The aim of this study was to evaluate the cytotoxicity and genotoxicity of the commercial formulation of glyphosate (Roundup® Control Max) at environmentally relevant concentrations and measure the potential effect of this herbicide over the cell capacity to repair DNA damage. HEK293 cells were exposed to 5 concentrations of Roundup® Control Max equivalent to 0.7; 7; 70; 700 and 3,500 μg/L glyphosate acid, for 1, 4 and 24 h. Cytotoxicity was quantified by the Trypan Blue staining method and by the MTT assay, while genotoxicity and evaluation of DNA damage repair kinetics were analyzed through the alkaline comet assay. In all treatments, cell viability was higher than 80%. The three highest glyphosate concentrations—70 μg/L, 700 μg/L, and 3,500 μg/L—increased levels of DNA damage compared to the control at the three exposure times tested. Finally, concerning the kinetics of DNA damage repair, cells initially exposed to 3,500 μg/L of glyphosate for 24 h were unable to repair the breaks in DNA strands even after 4 h of incubation in culture medium. The present study demonstrated for the first time that Roundup® Control Max may induce genetic damage and cause alterations in the DNA repair system in human embryonic kidney cells even at concentrations found in blood and breast milk of people exposed through residues of the herbicide in food, which values have been poorly assessed or not studied yet according to the existent literature.

Key words: Roundup; genotoxicity; DNA repair.

Introduction

Glyphosate is a systemic, non-selective, pre and post-emergence wide range herbicide[1,2] (Fig. 1). It was patented in 1971 by the American Company Monsanto and the commercial product was named "Roundup®".[3] This, as well as others glyphosate-based herbicides (GBH), contain glyphosate salt combined with surfactants, adjuvants and water.[4,5]

The use of products formulated with glyphosate continues to increase since its first introduction in the market in 1974, associated with agricultural practices like no-till cultivation of genetic modified herbicide-tolerant seeds, weed control and illegal crops.[6] However, the exponential growth in the use of this substance began in 1996 with the introduction of glyphosate-tolerant "Roundup-Ready" crop varieties (soybean, maize, canola, cotton, sugar beet and alfalfa), which are genetically modified to resist pulverizations with Roundup®.[5–7] Nowadays, uncountable GBH are registered in more than 130 countries worldwide and approved for their use in over 100 types of crops.[8] The United States, Canada, Brazil and Argentina are the major producers of glyphosate-tolerant genetically engineered seeds.[9,10]

The unrestricted, widespread, large-scale application of GBH in the last decades,[11] in the most diverse agricultural sectors and in urbanized areas,[12] leaded to its accumulation in the environment, edible product, and sanitary products such as diapers, gauze bandages and feminine hygienic products.[11,13,14] As a consequence, the health of aquatic and terrestrial animals,[12] including humans, is at risk and severely compromised.[15] Many experts consider glyphosate as the most sprayed and distributed chemical substance in human history.[5,6,13,16]

The mode of action of glyphosate is the selective inhibition of the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS). This enzyme interferes in the synthesis of chorismic acid, main precursor of aromatic amino acids Phenylalanine, Tyrosine and Tryptophan,[17,18] compromising the production of essentials proteins for the growth and survival of the plant.[1] This metabolic pathway is exclusive of plants, algae, bacteria and fungus metabolism.[12,19] EPSPS is non-existent in vertebrates, thus, it is supposed that glyphosate does not represent a risk for mammal's health, particularly humans.[20–23] However, several emerging evidences suggest that glyphosate or GBH, such as Roundup®, may have a negative impact in the biology of mammals through different mechanisms, including the capacity to generate genetic damage in eukaryotic cells.[2,24–29] It is important to highlight that the potential of a compound to cause genotoxicity and failures in

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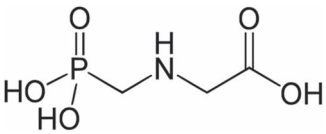

Fig. 1. Glyphosate molecular structure.

the DNA repairing systems, are among the criteria established by the International Agency for Research on Cancer (IARC) to classify chemical substances based on their carcinogenic risk.[30]

The interaction between glyphosate and genetic material has been reported by numerous researchers around the world, through analysis by different techniques and experimental designs, including in vivo and in vitro models.[31] In the year 2015, IARC classified glyphosate in group 2A as an agent "probably carcinogenic to humans", basing on "sufficient" evidence of carcinogenicity in experimental animals, "limited" evidence of carcinogenicity in humans and "strong" evidence of carcinogenic mechanisms such as genotoxicity and oxidative stress.[32]

Nowadays, it is widely known that DNA repair mechanisms play an essential role in the prevention of cancer through many pathways involving groups of repair enzymes that recognize different types of DNA damage.[33] DNA repair activity is a valuable biomarker of susceptibility to mutation and cancer, due to the fact that a high repair activity is related to a decrease of the chance of unrepaired damage when cells replicate and so to a decrease in potential mutations. Also, a high repair activity induces the synthesis of repair enzymes, which reflect exposure to DNA-damaging agents. Anyway, it can be concluded that a high repair activity is always essential for a correct cell function.[34]

The Comet assay is a method to detect genotoxicity that, in the last decades, has proven to be highly valuable to identify genotoxic compounds. This method not only detects DNA breaks, but also measures the DNA repair capacity in cells and tissues.[35] The easiest approach to measure DNA repair activity is treating the cells with a genotoxic agent, then remove it to facilitate the repair process and measure the remaining amount of damage at different times intervals.[34]

One of the first approaches to analyze the genotoxic effect of a substance in the biology of mammalian is the use of cells lines as an in vitro model, on which to expose the agent. Environmental exposure to Glyphosate occurs mainly through dermal contact and through the respiratory or digestive routes. In all cases, the first exposed are epithelial cells. HEK293 is a well-established epithelial cell line frequently used to evaluate cytotoxic effects.[36,37] Therefore, the aim of this study was to evaluate the cytotoxicity and genotoxicity of the commercial formulation of glyphosate (Roundup® Control Max) at environmentally relevant concentrations on HEK293 cells and subsequently measure the potential effect of this herbicide over the cell capacity to repair the DNA damage.

Materials and methods

Cell line and culture conditions

Human embryonic kidney cells (HEK293) from the laboratory of Molecular Biology, National University of Río Cuarto, were used for this study. The cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v fetal bovine serum (FBS), 1% antibiotic/antimycotic (10,000 U/mL penicillin, 10,000 μg/mL streptomycin, 25 μg/mL amphotericin B, Gibco®) and 1% v/v sodium bicarbonate, at 37 °C in a humidified atmosphere of 5% CO_2 in air.

When cells reached 70% to 80% confluence, were trypsinized (trypsin 0.5%, Sigma-Aldrich) and seeded at a concentration of 5×10^4 cells/well in a 24-well plate, with a final volume of medium of 800 μL/well. Cells were incubated in the same conditions for 24 h until confluent monolayers were formed. Then, the culture medium was removed and replaced with the 5 concentrations of Roundup® Control Max evaluated (dissolved in DMEM), maintaining the final volume of 800 μL/well and exposing the cells at different times (1, 4 and 24 h). A negative control (only DMEM) and a positive control (H_2O_2 10 μM dissolved in DMEM) were included.[38] All treatments were performed in duplicate. After exposure time, the culture medium was removed and cells were rinsed with phosphate buffer saline (PBS) 1× to eliminate remains of serum, metabolites or DMEM. Cell detachment was achieved through trypsinization using a 0.05% trypsin-EDTA solution (Thermo Fisher Scientific) for a period of 5 min. The trypsin reaction was halted by adding an equal volume of complete growth medium containing serum. Cells were then centrifuged at 300 × g for 5 min, the supernatant was removed, and the cell pellet was resuspended in 1× PBS at 6×10^5 cells/mL concentration to determine cell viability percentage and, subsequently, perform Comet assay.

Selection of suitable concentrations of Roundup® Control Max to be evaluated

In order to evaluate environmentally relevant concentrations, the maximum level allowed in workplaces (dermal and respiratory exposure) and the maximum level allowed in drinking water (digestive exposure) were included. The tested concentrations of Roundup® Control Max (contains 79.2% w/w of glyphosate monoammonium salt, plus adjuvants) were 0.7; 7; 70; 700 and 3,500 μg/L of glyphosate acid equivalent in culture medium. Highest concentration (3,500 μg/L), represents the occupational exposure limit (OEL) to glyphosate established by the United States Environmental Protection Agency in 2004[39]; second concentration (700 μg/L) is the maximum contaminant level (MCL) of glyphosate in drinking water legally enforceable to ensure its quality in USA, determined by EPA[40] and the three lowest concentrations were obtained as serial dilutions of the previous one.

Determination of cell viability by Trypan Blue exclusion test

The method of Trypan Blue dye exclusion was performed to the cell suspensions treated with each concentration of Roundup® for 1, 4 and 24 h and the controls to determine cell viability.[41] 50 μL of each cell suspension was mixed with 50 μL trypan blue solution (0.4% in PBS 1×) and a Neubauer chamber was used to cell counting. Viable (unstained) and death (blue stained) cells were counted, and results were expressed as percentage of viable cells (cell viability), both in controls and treatments.

Determination of cell viability by MTT assay

The MTT assay was performed following the method described by Mosmann[42] and modified by Denizot and Lang,[43] based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in a colored formazan salt by mitochondrial and cytosolic enzymes. Cell line was seeded at 5×10^3 cells/well in a 96 well plate and allowed to grow for 24 h at 37 °C and 5% CO_2. Then, the five tested concentrations of Roundup® were added to wells and incubated for 1, 4 and 24 h. Positive and negative controls were included. After, the medium was removed, the cells were rinsed with PBS prior to the addition of a MTT solution (5 mg/mL in DMEM 10% FBS) at a final concentration of 0.5 mg/mL,

and incubated for 3 h at 37 °C. The medium was replaced by 100 μL of dimethyl sulfoxide (DMSO) to dissolve the formazan crystals and the absorbance of this purple color was determined spectrophotometrically at 540 nm in a microplate reader (Bio-Rad, Hercules, CA, USA). Cell viability at different times of exposure (1, 4 and 24 h) was expressed as percentage (%) and compared against the optical density of the negative control (DMEM 10% FBS), considered as 100% of viability.

Quantification of DNA damage by single cell gel electrophoresis assay (Comet assay)

The alkaline single cell gel electrophoresis assay (Comet assay) was performed on those treatments that showed viability higher than 80% on Trypan Blue and MTT assays. The protocol followed the general guidelines proposed by Singh et al.[44] Cells previously exposed to the different concentrations of Roundup® at three times, and cells of the control wells, were collected. 75 μL of this cell suspensions were added to 150 μL of 1% low melting point (LMP) agarose at 37 °C in eppendorf tubes of 1,5 mL. The mixture was layered onto slides pre-coated with 1% of normal melting point (NMP) agarose, and then another layer of LMP agarose was added on top and, finally, covered with a coverslip. Once the agarose solidified, the coverslips were removed and the slides were carefully placed inside Coplin jars, immersed in a cold lysis solution (NaCl 2.5 M; EDTA 100 mM; Tris 10 mM; 1% Triton and 10% DMSO) at 4 °C for at least 24 h. After this time, the slides were transferred to an electrophoresis chamber containing an alkaline buffer pH 14 (NaOH 10 M; EDTA 200 mM). They were kept in this solution for 20 min, in absence of light (to prevent additional damage to DNA), at 4 °C. Electrophoresis was conducted at 1.0 V/cm with an amperage of ∼250 mA for 30 min. Slides were neutralized by dripping a solution of 0.4 M Tris (pH 7,5) onto their surface, the process was repeated 3 times every 5 min. Finally, the slides were stained with Ethidium Bromide (20 μg/mL) and observed using a fluorescence microscope at 400× (Zeiss) in dark. At least 200 nucleoids from each treatment were photographed at random; cells with non-detectable nuclei or "clouds" were not included. The images were then analyzed by the Comet Score 1.5®, Tri Tek Co software and the data was automatically transferred to Excel for further processing. The parameter used to infer DNA damage was Tail Moment.

Evaluation of the DNA repair kinetics in HEK293 cells grown in monolayer culture exposed to Roundup® Control Max

Based on the concentration of glyphosate that caused a statistically significant increase of DNA damage detected by Comet assay, the repair kinetics of HEK293 cells was assessed. Once again, 5×10^4 cells per well were seeded in 24-well plates with DMEM supplemented with 10% v/v FBS and 1× of antibiotic/antimycotic. The plates were incubated for 24 h at 37 °C and 5% CO_2 until confluent monolayers were formed (70%–80% confluence). Following the method described by Kwiatkowska et al.[25] and Woźniak et al.[29] the medium was replaced with Roundup® Control Max solutions at the concentrations that induced DNA strand damage. Negative control wells containing just DMEM-10% FBS v/v were included. After the incubation period, cells were rinsed 3 times with PBS 1× and resuspended in fresh medium. 75 μL aliquots of each cell suspension treatment were taken at time zero, 60, 120 and 240 min, and Comet assay was performed once more time. In sum, the repair kinetic of DNA damage was evaluated for 4 h. Analysis and classification of comets were carried out as described previously (Section 2.5).

Statistical analysis

All experiments were performed in duplicate. Data was analyzed using the Prism 5.0 software[45] and results were expressed as mean ± standard error. Kolmogorov-Smirnov test was performed to verify whether they follow a normal distribution and Bartlett's test to verify homogeneity of variance. Statistical analysis was performed by ANOVA, followed by Dunnett's post-hoc test. Non-parametric Kruskal-Wallis test and Dunn's multiple comparisons was used as a posteriori test for Comet assay, since data did not follow normal distribution. Spearman correlation coefficients were performed to examine possible concentration-response association. In all cases, the level of significance was set at $p \leq 0.05$.

Results

Determination of cell viability by Trypan Blue exclusion test

Cell viability was between 92.76% and 98.92% for cells exposed to glyphosate concentrations of 0.7–3,500 μg/L for 1 h, 98.57% for negative control and 94.25% for positive control (Fig. 2). As it is shown in the figure, the cell viability after 4 h treatment was between 98.22% and 99.77%; viability values were 97.92% and 98.67% for negative and positive controls, respectively. Cells treated for 24 h presented a viability of 89.21%–95.66% for those exposed to the different concentrations of glyphosate (0.7–3,500 μg/L), 98.09% for negative control and 90.47% for positive control. However, according to the analysis of variance (ANOVA), the differences in the values of viability between the three exposure times (1, 4 and 24 h), positive control (H_2O_2 10 μM) and the negative control were not statistically significant.

Determination of cell viability by MTT assay

Cell viability of negative controls was near to 100% at the three exposure times. Cells exposed to glyphosate concentrations between 0.7 and 3,500 μg/L for 1 h showed a viability of 80.43%–93.47% (Fig. 3). The viability values of those cells treated for 4 h was 97.73%–113.64%; and between 89.8%–97.96% for cells exposed for 24 h. ANOVA test showed that the differences of viability between treated cells, positive control (H_2O_2 10 μM) and negative control were not statistically significant.

Quantification of DNA damage by single cell gel electrophoresis assay (Comet assay)

Figure 4 represents Tail Moment values of HEK293 cells exposed to either five concentrations of glyphosate (0.7–3,500 μg/L) for 1, 4 and 24 h, medium culture (negative control) or to H_2O_2 10 μM (positive control). Kruskal-Wallis test and Dunn's multiple comparisons showed a statistically significant increase of DNA damage in cells exposed to three the highest concentration of glyphosate tested −70 μg/L ($p \leq 0.05$), 700 μg/L and 3,500 μg/L ($p \leq 0.001$)- compared to negative control. Similar response pattern was observed at the three times of exposure tested (1, 4 and 24 h).

Evaluation of the DNA repair kinetics in HEK293 cells grown in monolayer culture exposed to Roundup® Control Max

As shown in Fig. 5, after 240 min of post-incubation with culture medium, HEK293 cells were able to repair the DNA lesions provoked by previous exposure to 70 and 700 μg/L of glyphosate for 24 h. However, cells initially exposed to 3,500 μg/L of glyphosate

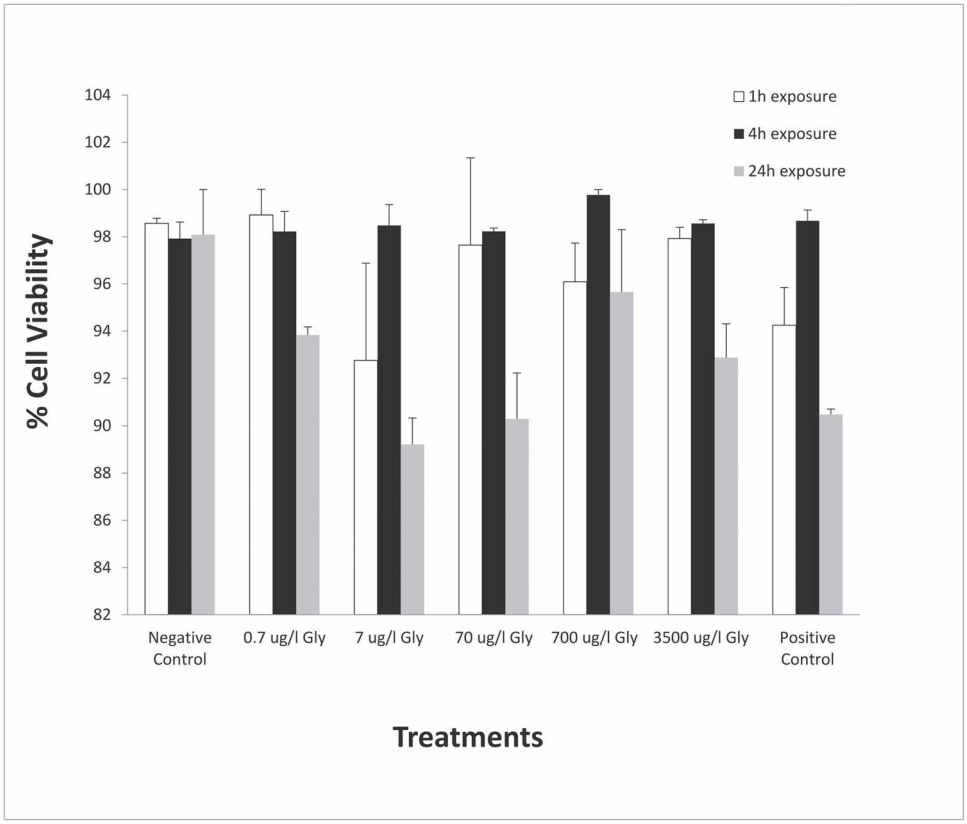

Fig. 2. Cell viability evaluation by Trypan Blue dye exclusion assay (%) in HEK293 cells exposed to: five concentrations of Glyphosate (0,7–7–70–700 and 3,500 μg/L) at different times (1, 4 and 24 h), H2O2 10 μM (positive control) and culture medium (negative control). Data expressed as mean ± SEM. Reference: Gly = Glyphosate.

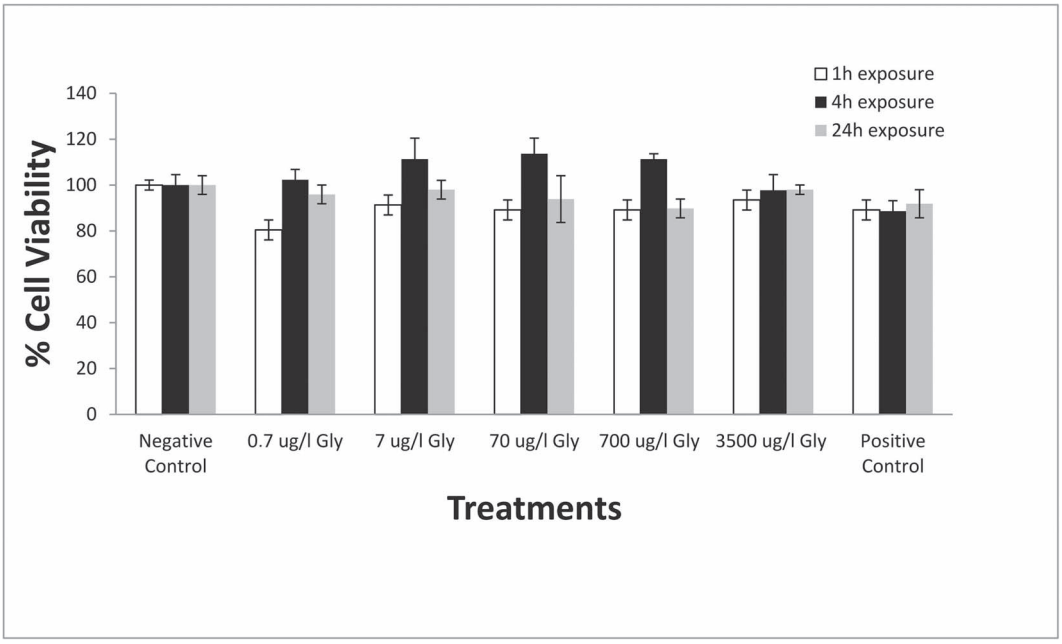

Fig. 3. Viability by MTT assay (%) of HEK293 cells exposed to: five concentrations of Glyphosate (0.7–7–70–700 and 3,500 μg/L) at different times (1, 4 and 24 h), H_2O_2 10 μM (positive control) and culture medium (negative control). Data expressed as mean ± SEM. Reference: Gly = Glyphosate.

could not repair the breaks in DNA strands after same time of incubation in culture medium. After post-incubation time, the difference in DNA damage values between the control group and the group exposed to the highest concentration (3,500 μg/L) was still statistically significant ($p \leq 0.01$), according to Kruskal-Wallis test and Dunn's multiple comparisons.

Discussion

MTT and Trypan blue exclusion tests showed that after 1, 4 and 24 h of HEK293 cells exposure to different concentrations of Roundup® Control Max (0.7–3,500 μg/L of glyphosate acid equivalent), there were not found statistically significant

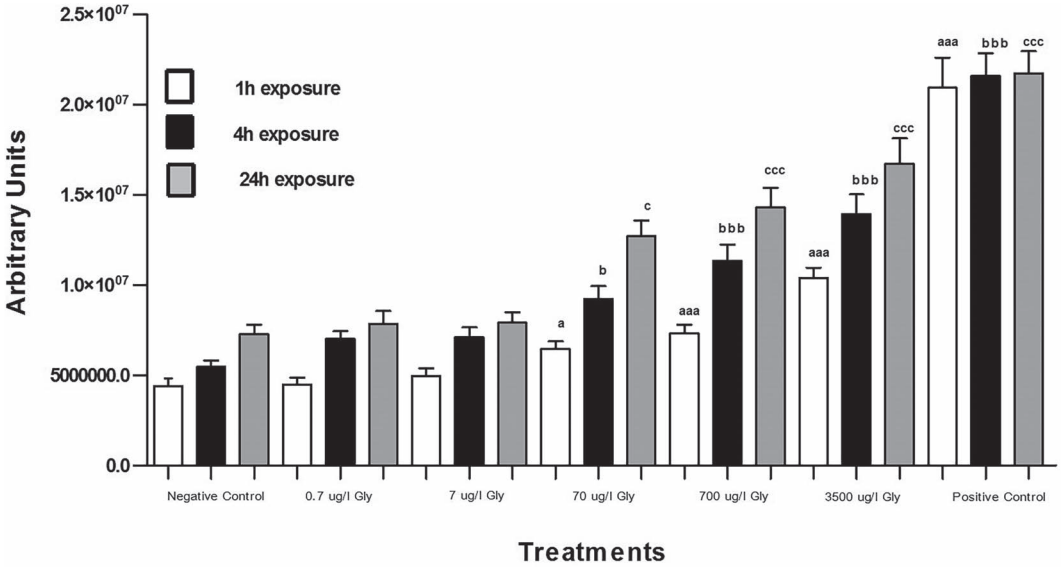

Fig. 4. Tail Moment (arbitrary units) of HEK293 cells exposed to: five concentrations of Glyphosate (0.7–7–70–700 and 3,500 μg/L) at different times (1, 4 and 24 h), H_2O_2 10 μM (positive control) and culture medium (negative control). Data expressed as mean ± SEM. ([a]) Statistically significant differences compared to negative control after 1 h of exposure; $^{a} = p \leq 0.05$ and $^{aaa} = p \leq 0.001$. ([b]) Statistically significant differences compared to negative control after 4 h of exposure; $^{b} = p \leq 0.05$ and $^{bbb} = p \leq 0.001$. ([c]) Statistically significant differences compared to negative control after 24 h of exposure; $^{c} = p \leq 0.05$ and $^{ccc} = p \leq 0.001$. Dunn's multiple comparisons. Reference: Gly = Glyphosate.

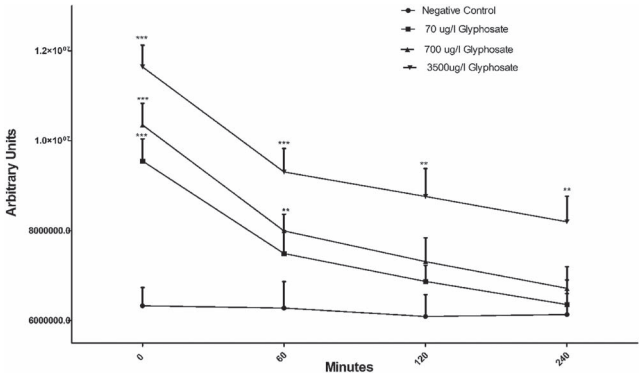

Fig. 5. Temporal evolution of repair kinetics for DNA damage in HEK293 cells after 24 h of exposure to: three concentrations of Glyphosate (70, 700 and 3,500 μg/L) and culture medium (control group). Repair kinetics was evaluated as a decrease on DNA damage after 60, 120 and 240 min of post-incubation (in culture medium) through comet assay, using Tale Moment as damage parameter (arbitrary units). Data expressed as mean ± SEM. (∗) Statistically significant differences compared to negative control at 0, 60, 120 and 240 min; ∗∗ = p ≤ 0.01 y ∗∗∗ = p ≤ 0.001. Dunn's multiple comparisons test.

differences in cell viability of experimental groups compared to the negative control group. These results agree whit those obtained by Woźniak et al.[29] who tested the commercial formulation of Roundup® 360 PLUS (containing 360 g/L of glyphosate as the potassium salt), which concentrations were to: 0.17–8,500 μg/L expressed as glyphosate. In this study, peripheral blood mononuclear cells (PBMCs) were exposed to the mentioned concentrations for 24 h and no statistically significant differences in cell viability were observed between experimental and control groups, except 8,500 μg/L. However, higher concentrations of glyphosate caused significant decrease of cell viability. It is worth mentioning that, in previous studies to this one (no published data), we observed a significant decrease in the same parameter at concentrations of 7,000 μg/L of glyphosate acid; a value close to the highest concentration tested by Woźniak et al.[29] (8,500 μg/L). However, Koller et al.[46] and Martinez et al.[47] demonstrated that the cytotoxic effects caused by Roundup® in epithelial cheek cells and in PBMCs, respectively, were more pronounced than that of pure glyphosate. In general, it is recognized that commercial glyphosate-based herbicides cause higher cytotoxicity than pure glyphosate.[22,48,49] These findings might be due to a considerable toxicity of surfactants and the presence of amine salts of glyphosate.[50]

Additional evidence that the cytotoxicity of commercial formulations containing glyphosate is higher than technical-grade glyphosate is supported by Townsend et al.[28] In this study, human Raji cells were exposed to concentrations of 17 μg/L–2,550 mg/L of technical-grade glyphosate (95% purity) for 24 h. No statistically significant differences in the cell viability of experimental groups were observed below 1,700 mg/L of glyphosate when compared to control groups. Even so, Raji cells showed a quick switch to an apoptotic profile shortly after the exposure to concentrations of 1,700 and 2,550 mg/L, provoking a significant drop in cell viability. These results prove that technical-grade glyphosate has a definitive cytotoxic nature in human cells at huge concentrations. A study performed by Kwiatkowska et al.[25] where they assessed the effects of technical-grade glyphosate (95% purity) on PBMCs cells after 24 h of exposure to concentrations of 1,275–1,700 mg/L and observed no statistically significant differences in cell viability, confirms the above. Therefore, Bonfanti et al.[51] suggested that the differences in glyphosate-based herbicides should be carefully considered by the authorities, since sub-lethal and/or long-term effects can be significantly modulated by the active ingredient salt type and concentration of the adjuvants. In addition, Myers et al.[5] recommended that these commercial formulations should be prioritized for inclusion in government-led toxicology testing programs such as the U.S. National Toxicology Program.

Comet assay was performed to evaluate genotoxicity. This technique is a sensitive and valuable tool for detecting genetic damage in single cells[44] that allows the identification of DNA single-strand breaks (SSB) and double-strand breaks (DSB), and to detect alkali-labile sites (ALS) as well.[25,29]

In the present study, HEK293 cells exposed to different concentrations of Roundup® Control Max at 3 times (1, 4 and 24 h) showed a viability superior to 80% and, therefore, they were all included in the genotoxicity assess by Comet assay. However, a statistically significant DNA damage was observed at the highest concentrations of glyphosate −70 μg/L ($p \leq 0.05$), 700 μg/L and 3,500 μg/L ($p \leq 0.001$)—and in the positive control group ($p \leq 0.001$), at the three times of exposure respect to negative control. Also, Spearman test showed that this increase in genetic damage had a concentration-response relationship ($r = 1$; $p \leq 0.01$).

These results of genetic damage obtained by Comet assay are perfectly in line with those of Alvarez-Moya et al.[24] and are partially consistent with previous findings by Woźniak et al.[29] Alvarez-Moya et al.[24] evaluated the effects of technical-grade glyphosate (96% purity) on human lymphocytes exposed to 119 μg/L–119 mg/L for 20 h, the results showed a statistically significant damage in all the experimental groups ($p < 0.01$), which suggest that glyphosate induced genotoxicity even at the lowest concentration of 119 μg/L. In accordance to our results and to other studies, these authors also reported a positive correlation of DNA damage with the increment of glyphosate concentrations.[52,53]

Woźniak et al.[29] assessed the genotoxic effect of Roundup® 360 PLUS in the concentration range from 0.17 to 1,700 μg/L in PBMCs exposed for 24 h through the comet assay. A significant DNA damage was determined from 850 μg/L concentration onwards, approximately 12 times the genotoxic concentration observed in the present study. Simultaneously, the active ingredient of the commercial formulation (glyphosate 95% purity) was evaluated in the concentration range of 85 μg/L–170 mg/L, statistically significant DNA damage was detected from 42,5 mg/L concentration onwards ($p < 0.05$), a genotoxic concentration 50 times higher than Roundup® 360 PLUS. Therefore, these results evidenced that technical grade glyphosate is less genotoxic than the Roundup®, which is supported by other recent reports as well.[25,28] However, in a study performed by Kašuba et al.[31] on HepG2 cells exposed to technical grade glyphosate (≤100% purity) at concentrations of 500, 2,910 and 3,500 μg/L for 24 h, no significant differences in genetic damage was observed between experimental and control groups when compared the percentage of DNA in the comet tail. The authors suggested that these results may be related to the different mechanisms of DNA damage infliction. As known, some compounds generate covalent adducts in DNA which can cause a DNA structure distortion and later obstruction in DNA replication; enhanced cell death in the form of apoptosis and cell cycle arrest in G2 phase. Therefore, they concluded that a significantly lower DNA damage can be a sign of possible adduct formation,[31] based on previous evidence about adduct formation of glyphosate in mouse liver and kidneys following in vivo exposure to this compound and to Roundup®, respectively.[54,55]

Our results highlight the capacity of Roundup® Control Max to cause genetic damage in human cells at a low concentration of 70 μg/L glyphosate acid, meaning, at equivalent concentrations found in blood (73.6 μg/L) and breast milk (76 μg/L) of people exposed mainly through the consumption of food containing pesticide residues.[56,57] The widespread application of glyphosate-based herbicides has provoked its accumulation in the environment and food products.[11] Therefore, the indirect exposure through food is a health concern for adults, children and infants caused by the risk to a dietary intake of significant amounts of glyphosate residues contained in contaminated food such as meat, fruits and vegetables.[58] Some studies had shown higher values of residues in food samples derived from genetically modified resistant crops when compared to unmodified ones.[59] Such residues have been found in products derived from cereals,[60] traditional and organic honey,[61] legumes,[62] beer,[63] and wine and fruit juices as well.[64] Krüger et al.[65] analyzed human urine samples and detected increased levels of glyphosate in samples of people consuming conventional food than people consuming predominantly organic food. In the same study, the authors also observed that glyphosate in urine of a generally healthy population was significantly lower than in urine from a chronically diseased population.[65]

The repair kinetics of DNA damage in HEK293 cells exposed to genotoxic concentrations (70, 700 and 3,500 μg/L glyphosate acid equivalent) of Roundup® Control Max for 24 h was assessed. Results showed that cells exposed to 70 and 700 μg/L were capable of repairing the damage induced to DNA after 240 min. However, cells initially exposed to the highest concentration (3,500 μg/L) could not fully repair the DNA strand breaks and the difference in genetic damage when compared with the control group was statistically significant ($p \leq 0.01$). These results coincide with those obtained by Woźniak et al.[29] who reported that PBMCs cells, previously exposed to Roundup® 360 PLUS 850 μg/L glyphosate equivalent, repaired the DNA single-strand breaks after 120 min post-incubation with fresh medium. Moreover, similarly to our study, they demonstrated that PBMCs were unable to completely repair the DNA damage caused by the highest concentrations of Roundup® tested (equivalent to 1,700 μg/L of glyphosate).

Marques et al.[33] have shown an effective repair process of DNA damage originated by Roundup®, nevertheless, DNA repair enzymes seemed to be susceptible to higher levels of this compound (41.76 μg/L glyphosate equivalent), disclosing another facet of the risk associated with the tested agrochemical. This study also suggested that damage at both the DNA and repair machinery contributes to an increase of cancer risk, enlightening that damage at the DNA repair machinery is as deleterious as DNA damage itself. The reason for this is that DNA repair system is a key factor in preventing severe genetic damage such as mutations, DNA strand breaks and chromosomal aberrations,[33] virtually amending all the damage before permanent genome change can occur.[34] Errors or mistakes in repair system may lead to irreparable DNA damage, triggering mutations due to the insertion of incorrect bases by DNA polymerases during replication.[29] When such mutations occur in oncogenes, suppressor genes or cell cycle control genes, they may cause multiple diseases including malignant tumors.[66–69] In consequence, DNA repair processes play a vital role to prevent cancer development[34] and progression because its deregulation can result in higher levels of genomic instability, increased mutation rates and promote intratumor heterogeneity.[70–73]

Conclusions

The present study demonstrated for the first time that Roundup® Control Max may induce genetic damage and cause alterations in the DNA repair system in cells derived from human embryonic kidney at daily exposure concentrations, which values have been poorly assessed or not studied yet according to the existent literature. Therefore, further investigations regarding the impact that glyphosate concentrations found in human samples (blood, urine and breast milk) might have on the integrity of genetic material are necessary. In addition, we consider extremely important that regulatory authorities acknowledge that glyphosate residues (and possibly Aminomethylphosphonic acid, one of the primary

degradation products of glyphosate, as well) detected in water and food products are reaching potentially damaging levels for human population.

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